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# Bacterial degradation of chlorobenzoates under contrasting environmental conditions

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**Chapter 7**

# Summary / Samenvatting

Microbes are of crucial importance for the removal of potentially harmful organic compounds from the environment. However, the prevailing conditions strongly determine the activity of the microbial populations involved. Because of the diversity and variability of these circumstances in natural systems, the fate of xenobiotics is difficult to predict. Chapter 1 provided some examples of the influence of growth conditions on the potential of micro-organisms to degrade halogenated aromatic compounds.

The (in)activity of physiologically different microbial groups is influenced particularly by the presence of appropriate electron acceptors. With chlorinated and aromatic growth substrates, the mechanisms of halogen removal and ring-cleavage differ significantly between oxic and anoxic conditions. These conditions are the result of on the one hand oxygen input from the air, by plant roots and algae, and on the other hand biotic oxygen consumption and abiotic oxidation processes. This means that in environments with changes in the (ground)-water-table or with a light-dark cycle activities of microbial groups using different metabolic mechanisms for energy generation are expressed sequentially or simultaneously (Fig. 1).

Lipophylic chloroaromatics like PCBs or chlorophenols can become particle-bound which facilitates their burial in anoxic sediments. In anoxic sediments, reductive removal of chlorine substituents is a commonly encountered microbial process necessary for the complete degradation of such compounds. Anoxic Biesbosch sediment slurries completely dehalogenated highly chlorinated benzoates (CBas) (Chapter 2). However, this depended largely on the order in which chlorines were removed: *ortho*-dechlorination of 2,3,5- or 2,3,6-triCBa resulted in the formation of 3,5-diCBa or 3-CBa, which were dechlorinated without difficulty. Alternatively, 2,6-diCBa or 2-CBa, both products from initial *meta*-dechlorination(s), were recalcitrant and slowed down or prevented complete degradation. Stimulation of initial *meta*-dechlorination was obtained by pre-acclimation to 3-CBa or 3,5-diCBa, whereas pre-acclimation to 2,5-diCBa or 2,4-diCBa led to *ortho*-dechlorination. This suggested that dechlorinating microbial subpopulations differing in preference for the ring-position of the chloro-substituent were selectively stimulated. Interestingly, also non-preincubated Biesbosch slurries preferentially catalyzed initial *ortho*-chlorine removal, possibly as the result of the history of sample-site pollution or specific incubation conditions. Controlling the abiotic incubation conditions to selectively stimulate anaerobic populations which dechlorinate at specific ring-positions can be of importance in determining the degree and rate of degradation of a range of chloro-aromatics.

Although photoheterotrophic bacteria are known to use many aromatic compounds for growth under anoxic conditions in the presence of light, data on the degradation of chlorinated aromatics by such microbes are few (Fig. 1). Light-exposed anoxic incubations of water and sediment samples in the presence of chlorobenzoates plus benzoate (Ba) resulted in the enrichment of a novel, 3-CBa- and 3-bromobenzoate-degrading photoheterotrophic bacterium, strain DCP3 (Chapter 3). Anaerobic photo-organohetero-

trophic growth by this strain of *Rhodopseudomonas palustris* on 3-CBa occurred with a $\mu_{max}$ of 0.03 $h^{-1}$, a maximum specific growth rate 6-10 times higher than that of *D.tiedjei* DCB1, an organism which reductively dechlorinates 3-CBa to Ba. For *Rhodopseudomonas* strain DCP3 a 3-CBa pathway via 3-chlorobenzoyl-CoA and benzoyl-CoA was proposed (Chapter 4). This was based on the fact that consumption rates by washed cells and CoA-ligase activities in crude cell-free extracts did not differ significantly between Ba or 3-CBa grown DCP3 cultures, and on the detection of Ba in 3-CBa grown cultures after hydrolysis of CoA-esters. In Ba- or 3-CBa-grown cultures and washed-cell suspensions, usage of 2-CBa, 3,5-diCBa, and a wide range of substituted non-halogenated benzoates (but not phenols or chloroethenes) apparently occurred via this pathway as well (Chapter 3, 4). Because strain DCP3 could not use these benzoates as single carbon sources the observations illustrate that some compounds can be biodegradable even when these do not induce functional metabolic pathways by themselves.

The development and persistence of microbial populations degrading xenobiotics are important in determining the fate of these compounds after release. In methanogenic Biesbosch slurries, the time period before detectable dechlorination of CBas occurred was several weeks (Chapter 2). This was explained by assuming slow exponential growth (observed $\mu \approx 0.004$ $h^{-1}$) of an autochthonous initially small bacterial population of approx. 10 cells/ml slurry with a low per-cell dechlorination rate (1 pmol/cell/day). The capability of *Rhodopseudomonas palustris* DCP3 to grow photoheterotrophically on 3-CBa as a single substrate was not initially present in the original enrichment material (Chapter 3). Probably this organism quickly adapted to growth on 3-CBa alone during cultivation in the laboratory: it was only after subculturing in the presence of Ba + 3-CBa that the dechlorinating phototrophic enrichment was able to grow on 3-CBa as a sole C-source. The high affinity of strain DCP3 for 3-CBa (affinity constant $K_s \approx 3$ $\mu$M) determined in chemostat culture indicates a role for this organism under natural conditions.

In the presence of oxygen, many (lower) chlorinated aromatics have been shown to be biodegradable. However, the influence of low oxygen concentrations, as found in anoxic-oxic interfaces, on the degradation rates of xenobiotics has not been studied thoroughly so far. At controlled oxygen concentrations below 47 $\mu$M, 2,5-diCBa consumption rates went down significantly in *Pseudomonas* JB2 chemostat cultures (Chapter 5). This was probably caused by a low oxygen affinity of the cells during oxidation of 2,5-diCBa. Indeed, the affinity constants for oxygen ($K_m(O_2)$) were 19 and 28 $\mu$M during growth on Ba or 2,5-diCBa, respectively. Only the apparent affinity constant for the limiting substrate 2,5-diCBa ($K_m$(2,5-diCBa)) was observed to improve: *Pseudomonas* JB2 variants with lowered $K_m$ values (from 1 mM down to 40 $\mu$M) emerged during 2,5-diCBa-limited chemostat experiments (Chapter 5).

Generally for aerobic bacterial growth on chlorinated aromatic compounds a number of specialized enzymes is needed. However, the presence of non-chlorinated substrates may suppress the synthesis of these enzymes. The preferential use of easier degradable substrates over CBas by *Pseudomonas* JB2 was shown with mixtures of Ba plus 2,5-diCBa. In batch culture sequential consumption of 2,5-diCBa after Ba depletion occurred, whereas in C- or N-limited chemostat cultures consumption of 2,5-diCBa was suppressed or incomplete, respectively, during growth on Ba (Chapter

5). This can also have phenotypic effects, as in batch cultures of *Pseudomonas* JB2 grown on non-chlorinated substrates, high numbers of mutants which had lost the capability to use *ortho*-chlorinated benzoates arose even in the presence of 2,5-diCBa as an additional substrate (Chapter 6). Possibly these *ortho*-minus mutants had lost a plasmid or transposon on which genes coding for CBa pathways are most often located. *Pseudomonas* JB2 wild-type (WT) and its *ortho*$^-$ mutant, which showed higher specific growth rates on Ba and 3-CBa, co-existed in batch as well as in chemostat cultures on mixtures of Ba plus CBas. This implies that on substrate mixtures the capabilities of the culture are maintained, but that an initially phenotypically homogeneous population splits into specialized subpopulations. The observation that dehalogenating bacteria can not compete successfully for simple non-chlorinated compounds suggests that in natural C-limited environments, competitive non-dehalogenating subpopulations may stimulate the removal rate of chloro-organics by rapidly consuming simple alternative substrates, leaving only the chlorinated compound as a growth substrate for the dehalogenating microbes.

In view of the different degradative possibilities and responses under different conditions, computations predicting the fate

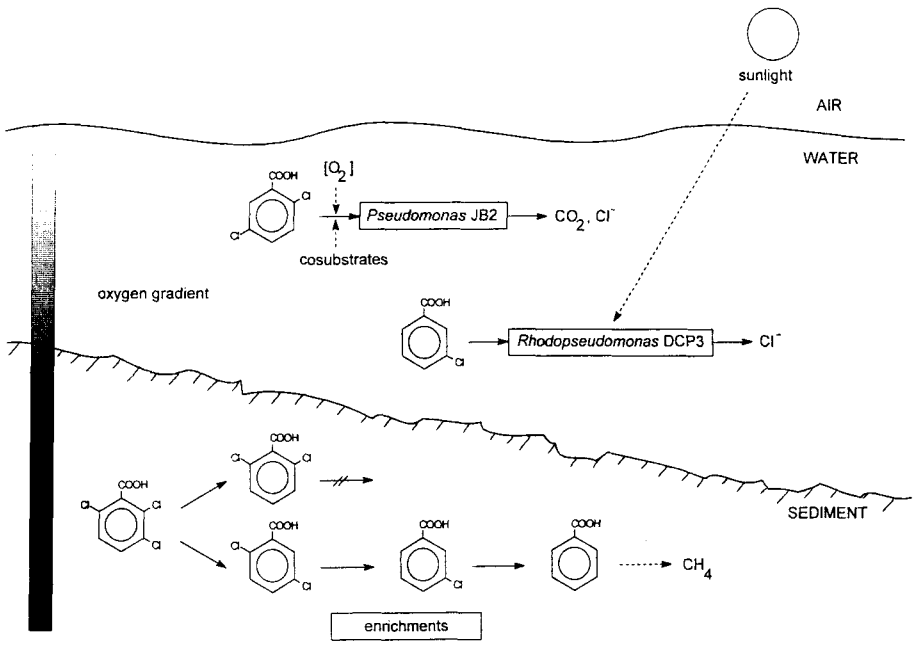


Figure 1. Degradation of chlorobenzoates in a hypothetical ecosystem including the different types of microbes studied in this thesis.

of a certain compound in an engineered ecosystem, e.g. a waste-water plant, should include the history (which influences the size and possibilities of degrading populations), the actual growth conditions (which influence the activities of different microbial groups), and the degradation rates found in the laboratory under the prevailing conditions. For natural systems the high heterogeneity and variability in environmental conditions make such calculations more difficult. In addition, it is not yet clear which contribution a specific microbial group makes to the breakdown of compounds in a polluted system. This applies e.g. to photoheterotrophic dechlorinating bacteria. However, their survival during oxic periods would certainly make such organisms of importance in alternating oxic/anoxic environments. In illuminated waste-water treatments in general, photoheterotrophic bacteria are ubiquitous and likely to be involved in the degradation of (chloro)aromatics.

Microörganismen zijn zeer belangrijk voor het verwijderen van mogelijk schadelijke organische verbindingen uit het milieu. De actuele condities sturen de activiteit van de betrokken microbiële populaties echter sterk. Aangezien de omstandigheden in natuurlijke systemen sterk variëren met plaats en tijd, valt het lot van xenobiotica moeilijk te voorspellen. Hoofdstuk 1 gaf enkele voorbeelden van de invloed van groeiomstandigheden op het vermogen van microörganismen om gehalogeneerde aromatische verbindingen af te breken.

De (in)activiteit van fysiologisch verschillende microbiële groepen wordt in het bijzonder beïnvloed door de aanwezigheid van geschikte electron-acceptoren. Bij gechloreerde of aromatische groeisubstraten verschillen de mechanismen van halogeen-verwijdering of ringsplitsing aanzienlijk tussen omstandigheden met en zonder zuurstof. Deze omstandigheden zijn het gevolg van enerzijds zuurstofleverantie vanuit de lucht, door plantewortels of algen, en anderzijds biotische zuurstofconsumptie of abiotische oxidaties. Dit betekent dat in milieu's met (grond)waterstandswisselingen of met een licht/donker-cyclus opeenvolgende of gelijktijdige activiteiten kunnen voorkomen van microbiële groepen met verschillende manieren van energiegenerering (Fig. 1).

Lipofiele chlooraromaten zoals PCB's of chloorfenolen kunnen zich binden aan partikels en daardoor gemakkelijker worden begraven in zuurstofloze sedimenten. In anoxische sedimenten is de reductieve verwijdering van chlooratomen een veel waargenomen microbieel proces, noodzakelijk voor de complete afbraak van dergelijke verbindingen. Zuurstofloze moddermengsels van Biesbosch-sediment dehalogeneerden hooggechloreerde chloorbenzoaten (CBas) geheel (Hoofdstuk 2). Dit hing echter sterk af van de volgorde waarin de chlooratomen werden verwijderd: *ortho*-dechlorering van 2,3,5- of 2,3,6-triCBa resulteerde in 3,5-diCBa of 3-CBa, die zonder probleem werden gedechloreerd. 2,6-diCBa of 2-CBa ont-staan uit initiële *meta*-dechloreringen werden echter niet verder gedechloreerd en vertraagden of verhinderden volledige afbraak. Stimulering van initiële *meta*-dechlorering werd verkregen door pre-acclimatie aan 3-CBa of 3,5-diCBa, terwijl pre-acclimatie aan 2,5- of 2,4-diCBa leidde tot *ortho*-dechlorering. Kennelijk werden dechlorerende subpopulaties die verschilden in voorkeur voor de ringpositie van de chloorsubstituent selectief gesti-